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# Extracellular Synthesis of Crystalline Silver Nanoparticles and Molecular Evidence of Silver Resistance from *Morganella* sp.: Towards Understanding Biochemical Synthesis Mechanism

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*There has been significant progress in the biological synthesis of nanomaterials. However, the molecular mechanism of synthesis of such bio-nanomaterials remains largely unknown. Here, we report the extracellular synthesis of crystalline silver nanoparticles (AgNPs) by using* Morganella *sp., and show molecular evidence of silver resistance by elucidating the synthesis mechanism. The AgNPs were* $20 \pm 5$ *nm in diameter and were highly stable at room temperature. The kinetics of AgNPs formation was investigated. Detectable particles were formed after an hour of reaction, and their production remained exponential up to 18 h, and saturated at 24 h.* Morganella *sp. was found to be highly resistant to silver cations and was able to grow in the presence of more than 0.5 mM* $AgNO_3$*. Three gene homologues viz.* silE, silP *and* silS *were identified in silver-resistant* Morganella *sp. The homologue of* silE *from* Morganella *sp. showed 99% nucleotide sequence similarity with the previously reported gene,* silE*, which encodes a periplasmic silver-binding protein. The homologues of* silP *and* silS *were also highly similar to previously reported sequences. Similar activity was totally absent in closely related* Escherichia coli*; this suggests that a unique mechanism of extracellular AgNPs synthesis is associated with silver-resistant* Morganella *sp. The molecular mechanism of silver resistance and its gene products might have a key role to play in the overall synthesis process of AgNPs by* Morganella *sp. An understanding of such biochemical mechanisms at the molecular level might help in developing an ecologically friendly and cost-effective protocol for microbial AgNPs synthesis.*

## Introduction

The chemical methods for the synthesis of metallic nanoparticles are quite successful and have been well studied.[1–3] However, the biological synthesis of such nanomaterials has gained significant interest due to the use of mild experimental conditions of temperature, pH, and pressure. If harnessed to their full potential, biological synthesis could present extra advantages over chemical methods such as higher productivity and lower cost. One new dimension in the metal–microbial interaction has emerged in the last few years, in that the synthesis of metallic nanoparticles have been reported from bacteria, yeast, fungi, and other biological sources.[4–10] AgNPs have received considerable attention due to their attractive physicochemical properties. The surface plasmon resonance and large effective scattering cross section of individual AgNPs make them ideal candidates for molecular labeling.[3] The existing physical methods for AgNPs synthesis, such as gas condensation and irradiation by ultraviolet or $\gamma$ radiation, are usually associated with a low production rate and high expense.[11,12] Further, the large-scale synthesis of silver nanomaterial suffers from issues such as polydispersity and stability, especially if the reduction is carried out in aqueous media. Therefore the extracellular biological synthesis of AgNPs could be an attractive and ecologically friendly alternative method for the preparation of large quantities because it offers the advantage of easy downstream processing. Moreover, bacteria are easy to handle and can be manipulated genetically without much difficulty. Considering these advantages, a bacterial system could prove to be an excellent alternative for the extracellular synthesis of AgNPs.

Ionic silver is highly toxic to most bacterial cells and has long been used as a potent bactericidal agent.[13] However, several silver-resistant bacterial strains have been reported and even shown to accumulate AgNPs in their periplasmic space.[4,14,15] Microbes from silver-rich habitats such as silver mines have been explored for the synthesis of AgNPs.[4] However, in the recent past, habitats like buttermilk have also been

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tapped for microbial synthesis of silver and gold nanoparticles;[16] this suggests that microbes from unusual and nonsilver-rich habitats can also be explored for AgNP synthesis. The rationale in using silver-resistant microorganisms for AgNP production is clear;[4,17] however, a plausible biochemical mechanism for metal-ion reduction and nanoparticle production has remained elusive. It was recommended to study the silver-resistance mechanism to understand the overall synthetic process of AgNPs from silver-resistant bacteria,[4,18] but no such information with respect to AgNPs synthesis is available to date. The mechanism that allows silver-resistant microbes to survive in a silver-rich environment was not properly understood until Gupta and co-workers studied the plasmid-mediated silver-resistance mechanism of *Salmonella*.[19] This functions as a cellular efflux pumping system that protects the cytoplasm against toxic concentrations of silver, and a small periplasmic silver-binding protein that binds silver specifically at the cell surface. It might be possible that the silver-resistance machinery could be playing a key role in the synthesis of nanoparticles. As a step towards understanding the biochemical synthesis, we address this issue and have identified the gene homologues that could play a major role in the synthesis process of AgNPs in silver-resistant *Morganella* sp.

## Results

### Identification of bacterium

The bacterium was originally isolated from the insect gut and was identified as *Morganella* sp. It belongs to the family Enterobacteriaceae. The identification of the isolate was carried out by 16S rRNA gene sequencing after preliminary biochemical analysis. The 1.5 kb sequence of 16S rRNA gene showed 99% similarity with *Morganella* sp. and was also supported by biochemical identification. The 16S rRNA gene sequence was deposited in the GenBank with the accession number EF525539.

### Extracellular synthesis of AgNPs from *Morganella* sp.

The bacterium was grown in LB broth without added NaCl for 15 h at 37 °C, and then an aqueous $AgNO_3$ solution at a final concentration of 5 mM was added. It was further allowed to incubate for 20 h at 37 °C. After removing bacterial cells by centrifugation, the supernatant was a clear brown homogenous suspension of AgNPs, and was analyzed by UV–visible spectroscopy. Figure 1 A shows the characteristic surface plasmon resonance (SPR) at about 415 nm for AgNPs[20] obtained from *Morganella* sp., which was absent in all appropriate simultaneously run controls. The AgNP production was not detected even in a closely related genus of the same family of *E. coli*, which indicates that the phenomenon could be specific to

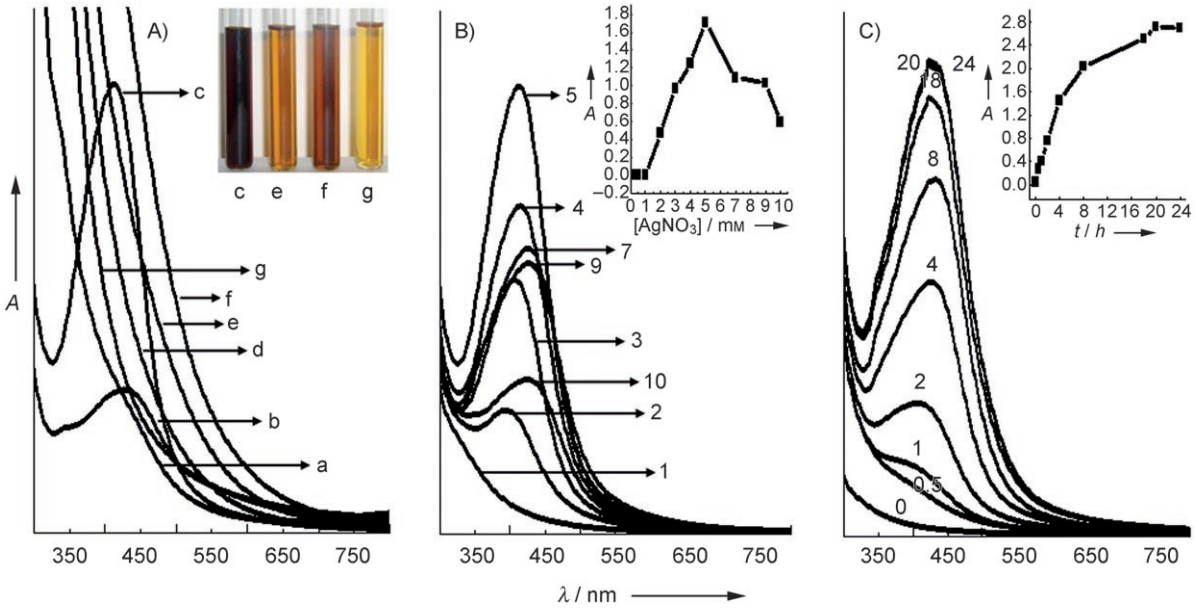


**Figure 1.** A) UV/Vis spectra recorded from the culture supernatant that shows the production of AgNPs from *Morganella* sp. Curve a, b and c shows AgNPs production after the addition of aqueous $AgNO_3$ solution to the overnight-grown *Morganella* sp. after 0 h, 1 h and 20 h of reaction, respectively. While curves d and e were recorded after the addition of aqueous $AgNO_3$ solution only to the LB broth (without added NaCl) after 1 h and 20 h of reaction, respectively. Curve f was recorded (without diluting supernatant) after the addition of aqueous $AgNO_3$ solution to the overnight-grown *Escherichia coli*. Curve g was recorded from the culture supernatant of *Morganella* sp. without added aqueous $AgNO_3$ solution after 24 h of growth. Inset Figure shows images of color of solution of curves c, e, f and g. B) UV/Vis spectra that were recorded from culture supernatant from different concentrations of $AgNO_3$ that were added to overnight-grown *Morganella* sp. The numbers given to the respective curves represent the concentration (mM) of the added aqueous $AgNO_3$ solution. Inset graph shows the absorbance of AgNPs that were produced against respective concentrations of added aqueous $AgNO_3$ solution. C) UV/Vis spectra showed the time-dependent kinetics of AgNPs production. The numbers over respective peaks indicate the time at which the spectrum was recorded. Inset graph shows the saturation curve of AgNPs production over a period of 24 h.

  

silver-resistant *Morganella* sp. Production optimization of AgNPs was performed with respect to concentrations of $Ag^+$ ions, which ranged from 1 to 10 mM. It clearly showed an increase in the intensity of the SPR up to 5 mM concentration, and an intensity decrease at higher concentrations (Figure 1B). The results clearly indicated that 5 mM concentration of $Ag^+$ ions was most appropriate for the synthesis of AgNPs from *Morganella* sp. (Figure 1B inset). The kinetics of AgNP production were studied by periodically analyzing samples at different time intervals after adding $Ag^+$ ions to the culture flask of *Morganella* sp. Figure 1C shows the progression of AgNPs synthesis over time. The intensity of SPR clearly increased with the time progression. Detectable nanoparticles appeared after an hour of adding salt to the culture media and their production remained exponential up to 18 h. The nanoparticle production was saturated at 24 h (Figure 1C inset). After harvesting bacterial cells, the culture supernatant was examined for its ability to produce AgNPs. UV–visible analysis of the culture supernatant also showed the formation of AgNPs (data not shown).

### Characterization of AgNPs

Transmission electron microscopy (TEM) images of AgNPs that were synthesized by *Morganella* sp., indicated that the nanoparticles were in the size range of ~20 nm and were spherical in shape (Figure 2A and B). In addition to 5 mM, the TEM analysis of AgNPs that were prepared from 2 and 10 mM $Ag^+$ solutions showed no significant change in the shape and the size of nanoparticles (Supporting Information). Selected area electron diffraction (SAED) spots that corresponded to the [111], [200], [220], and [311] planes of the face-centered cubic (fcc) structure of elemental silver[21] are clearly seen in Figure 2D. The high-resolution TEM (HRTEM) image (Figure 2C) shows the d spacing of 2.02 Å, which matches with the [200] plane of AgNPs. The X-ray diffraction (XRD) pattern (Figure 3A) shows intense Bragg's reflections that can be indexed on the basis of the fcc structure of silver.[21] The XRD pattern thus obtained clearly shows that the synthesized AgNPs by the *Morganella* sp. were crystalline in nature. A Fourier transform infra-red (FTIR) spectrum (Figure 3B) of AgNPs shows the presence of two absorption bands centered at about 1550 and 1650 $cm^{-1}$ (amide II and amide I bands respectively); this indicates the presence of proteins on the surface of the AgNPs that appear to be acting as capping and stabilizing agents. Chemical analysis of the AgNPs was carried out by X-ray photoelectron spectroscopy (XPS). Figure 3C and D show the carbon 1s and silver 3d core levels, respectively. The C 1s core level spectrum could be decomposed into three chemically distinct components that are centered at 281.3, 285.0 and 288 eV. The low binding energy peak at 281.3 eV is attributed to the presence of aromatic carbon atoms that are present in amino acids from proteins that are bound to the surface of silver nanoparticles.[22] The high binding energy peak at 288.0 eV is attributed to electron emission from carbon atoms in carbonyl groups (aldehydic or ketonic carbon) that are present in proteins that are bound to the nanoparticles surface.[23,24] The C 1s component that is centered at 285.0 eV is due to electron emission from an adventitious carbon atom that is present in the sample. The Ag 3d core level spectrum could be decomposed into a single spin-orbit pair (spin-orbit splitting ~6.0 eV) with $3d_{5/2}$ and $3d_{3/2}$

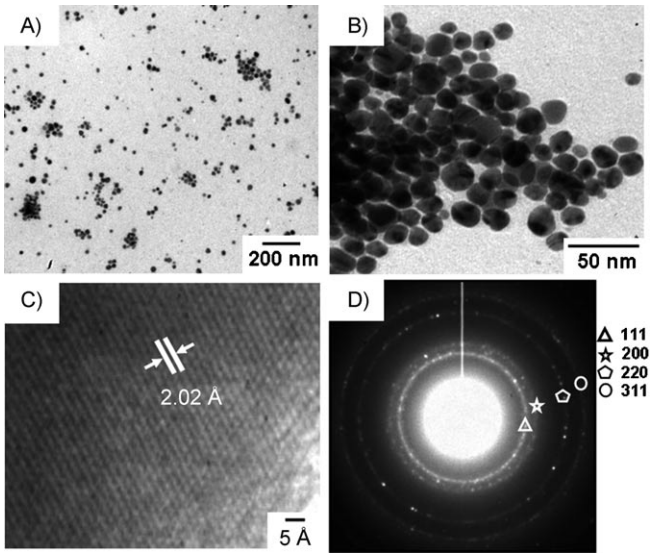


**Figure 2.** A) and B) Transmission electron microscopy images of AgNPs from *Morganella* sp. at different magnifications. C) HRTEM image showing characteristic *d* spacing for the [200] plane. D) Selected area electron diffraction showing the characteristic crystal planes of elemental silver.

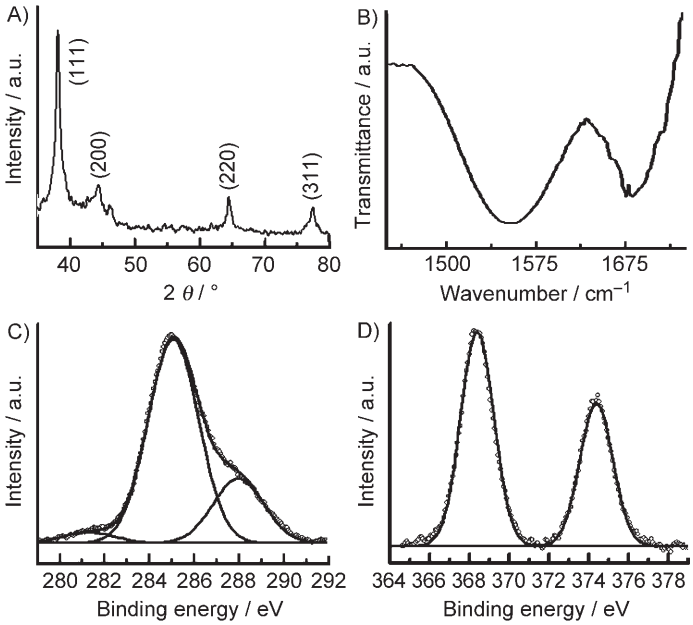


**Figure 3.** A) XRD spectra of AgNPs synthesized by using *Morganella sp*. showing [111], [200], [220], and [311] planes, which are characteristic of crystalline silver. B) FTIR spectrum recorded from purified AgNPs showing bands at about 1550 and 1650 corresponding to amide II and amide I respectively; this suggests the presence of capping proteins on AgNPs. XPS spectra of AgNPs showing the C) C 1s and D) Ag 3d core level spectra recorded from *Morganella* sp. The C 1s spectrum is resolved into three components (281.3, 285.0 and 288 eV) and the Ag 3d spectrum is resolved into two components (368.3 and 374.3 eV).

  

binding energies (BEs) of 368.3 and 374.3 eV, respectively; these agree with values that have been reported for metallic silver.[25] The energy dispersive analysis of X-rays (EDAX; Supporting Information) shows the presence of elemental silver in the sample.

### Cloning and sequence analysis of *sil* homologues

The phenotypic evidence of silver resistance was studied by growing *Morganella* sp. at and above the determined minimum inhibitory concentration of silver ions of silver-sensitive *E. coli*.[26] *Morganella* sp. was grown in the presence of different concentrations of $Ag^+$ ions that ranged from 0.1 to 1 mM. It was found that the bacterium was able to grow in more than 0.5 mM concentration of $Ag^+$ ions in LB broth without added NaCl, which is more than 500% more tolerant than sensitive *E. coli*.[26] This clearly indicated that the bacterium was highly resistant to $Ag^+$ ions. The genotypic evidence was studied by identifying three major gene homologues, namely *silE*, *silP* and *silS* of the putative silver-resistance machinery in *Morganella* sp. These three *sil* gene homologues were characterized by using polymerase chain reaction (PCR), cloning, and sequencing. Figure 4 shows the PCR products of *silE*, *silP*, and *silS* homologues from *Morganella* sp. These band sizes of PCR products corresponded to the expected size of reported *silE*, *silP* and *silS* of silver resistance. On the basis of partial sequence analysis, the *silE* homologue was identified as a periplasmic silver-binding protein; the *silP* homologue was identified as a cation-transporting P-type ATPase, and the *silS* homologue was identified as a two-component membrane sensor kinase of the silver-resistance mechanism. Figure 5 shows the multiple sequence alignment of the *silE* homologue with related sequences that were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) database. The multiple nucleotide sequence alignment data clearly showed that the *silE* homologue from *Morganella* sp. was 99% similar with the reported *silE*,[19] which indicated that it could be playing a similar role in silver-resistant *Morganella* sp., too. Gupta and co-workers have clearly shown a specific interaction between the periplasmic silver-binding protein that is encoded by *silE* and $Ag^+$ ions.[19] Similarly, multiple nucleotide sequence alignment data (Supporting Information) of *silP* and *silS* homologues with reported sequences showed that they were also highly similar with reported sequences,[19] and could also be playing a similar role in silver-resistant *Morganella* sp. In addition, multiple alignment of in-silico-translated amino acid sequences of *sil* gene homologues also showed high similarity with reported sequences (Supporting Information). The sequences of *sil* gene homologues were deposited to the GenBank with accession numbers EF525536, EF525537 and EF525538.

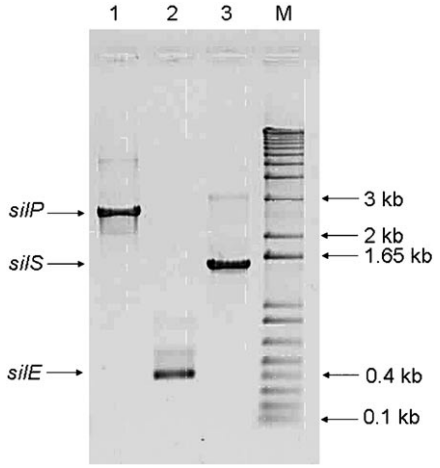


**Figure 4.** PCR amplification products of *sil* gene homologues resolved on ethidium-bromide-stained 1% agarose gel. Lanes 1, 2 and 3 contained *silP*, *silE* and *silS* homologues respectively, as indicated by arrows on left. Lane M contained DNA size markers, whose sizes are indicated on the right.

## Discussion

Silver nanoparticles have a wide range of applications from electronics to medicine. They are used as intercalating material,[18] optical receptors,[3] chemical catalyst for biolabeling,[27] and as an antimicrobial agent.[28] Although physical and chemical methods of synthesis of AgNPs are widely used, there has been growing interest in developing biological synthetic protocols for these particles. This is mainly due to the advantages that biological synthesis promises over physical and chemical synthesis such as high yield, low cost, high stability in liquid suspension, and environmental friendliness. Attempts have been made to synthesize such nanomaterials from such microorganisms as bacteria,[4] fungi,[5] yeast,[17] and blue-green algae,[9] however, a bacterial system is preferred for the production of such bio-nanoconjugates over eukaryotic microorganisms mainly due to reduced time of reaction, ease of handling, and easy genetic manipulation. Moreover, studies on one bacterium can be easily extrapolated to others. Methods for the extracellular synthesis of nanomaterials are particularly desirable because they offer the advantage of downstream processing. Even though, there are reports that evidence the extracellular synthesis of AgNPs from microbes,[5,9,17] and intracellular accumulation in bacteria,[4] no report exists that shows extracellular synthesis of AgNPs from bacteria. Considering the above-mentioned facts, extracellular AgNPs synthesis from silver-resistant *Morganella* sp. that was observed in this study would be a significant advance in this field. The best exploitation of any biological system for such a purpose is only possible when the mechanism of synthesis of such nanomaterials is properly understood. It is noteworthy that in all previous reports,[4,17] microorganisms that were utilized for AgNP synthesis were silver resistant. However, the mechanism of synthesis in relation to AgNPs still remains elusive. Moreover, *Morganella* sp. that was used in this study for AgNP synthesis was also found to be silver resistant. Taken together, these facts made the basis for studying the role of the silver-resistance machinery in AgNPs that are produced by silver-resistant microorganisms. It is a known fact that microbes attain genetic and biochemical mechanisms to eliminate highly toxic silver ions in

  

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Mor  ATGAAAAATATCGTATTAGCATCCTTGCTGGGCTTTGGCTTAATTTCTTCGGCCTGGGCC 60
Sty  ATGAAAAATATCGTATTAGCATCCTTGCTGGGCTTTGGCTTAATTTCTTCGGCCTGGGCC 60
Smar ATGAAAAATATCGTATTAGCATCCTTGCTGGGCTTTGGCTTAATTTCTTCGGCCTGGGCC 60
Sen  ATGAAAAATATCGTATTAGCATCCTTGCTGGGCTTTGGCTTAATTTCTTCGGCCTGGGCC 60
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Mor  ACTGAAACCGTGAATATCCATGAGCGGGTCAACAATGCACGGGCACCTGCTCACCAGATG 120
Sty  ACTGAAACCGTGAATATCCATGAGCGGGTCAACAATGCACAGGCACCTGCTCACCAGATG 120
Smar ACTGAAACCGTGAATATCCATGAGCGGGTCAACAATGCACAGGCACCTGCTCACCAGATG 120
Sen  ACTGAAACCGTGAATATCCATGAGCGGGTCAACAATGCACAGGCACCTGCTCACCAGATG 120
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Mor  CAGTCTGCTGCGGCTCCTGTCGGGATCCAGGGGACTGCACCTCGTATGGCCGGTATGGAC 180
Sty  CAGTCTGCTGCGGCTCCTGTCGGGATCCAGGGGACTGCACCTCGTATGGCCGGTATGGAC 180
Smar CAGTCTGCTGCGGCTCCTGTCGGGATCCAGGGGACTGCACCTCGTATGGCCGGTATGGAC 180
Sen  CAGTCTGCTGCGGCTCCTGTCGGGATCCAGGGGACTGCACCTCGTATGGCCGGTATGGAC 180
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Mor  CAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGGGTCGGCGGATGCGCAC 240
Sty  CAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGGGTCGGCGGATGCGCAC 240
Smar CAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGGGTCGGCGGATGCGCAC 240
Sen  CAGCATGAACAGGCCATTATTGCTCATGAAACCATGACGAACGGGTCGGCGGATGCGCAC 240
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Mor  CAGAAAATGGTGGAAAGTCATCAGAGGATGATGGGAAGTCAGACCGTTTCCCCTACCGGG 300
Sty  CAGAAAATGGTGGAAAGTCATCAGAGGATGATGGGAAGTCAGACCGTTTCCCCTACCGGG 300
Smar CAGAAAATGGTGGAAAGTCATCAGAGGATGATGGGAAGTCAGACCGTTTCCCCTACCGGG 300
Sen  CAGAAAATGGTGGAAAGTCATCAGAGGATGATGGGAAGTCAGACCGTTTCCCCTACCGGG 300
     ************************************************************

Mor  CCGTCGAAGTCATTAGCGGCAATGAATGAGCATGAAAGAGCTGCAGTTGCCCATGAATTT 360
Sty  CCGTCGAAGTCATTAGCGGCAATGAATGAGCATGAAAGAGCTGCAGTTGCCCATGAATTT 360
Smar CCGTCGAAGTCATTAGCGGCAATGAATGAGCATGAAAGAGCTGCAGTTGCCCATGAATTT 360
Sen  CCGTCGAAGTCATTAGCGGCAATGAATGAGCATGAAAGAGCTGCAGTTGCCCATGAATTT 360
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Mor  ATGAATAACGGTCAGTCTGGCC 382
Sty  ATGAATAACGGTCAGTCTGGCC 382
Smar ATGAATAACGGTCAGTCTGGCC 382
Sen  ATGAATAACGGTCAGTCTGGCC 382
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**Figure 5.** Multiple nucleotide sequence alignment of *silE* homologue from *Morganella* sp. shows a high percentage similarity with related sequences from the NCBI database. Mor: *Morganella* sp. (EF525536), Sty: *Salmonella typhimurium* (AF067954), Sma: *Serratia marcescens* (BX664015) and Sen: *Salmonella enterica* (AY009392). The numbers in parenthesis indicates the GenBank accession numbers of respective sequence.

the environment through silver-resistance machinery. We have also shown phenotypic and genotypic evidence of silver resistance, and believe that it could represent significant progress towards understanding the overall mechanism of AgNP synthesis from silver-resistant microbes.

The particles were characterized and analyzed in different ways, and were found to be composed exclusively of metallic silver. Most of the particles were spherical in shape, had size of 20 nm, were highly stable, and showed no visible aggregation and precipitation at room temperature over a period of six months. UV–visible analysis of a six-month-old sample also supported the visible observation, and showed no change in its characteristic SPR peak compared to a freshly prepared sample (Supporting Information). To validate the probable effect of various concentrations of $AgNO_3$ on the shape and the size of nanoparticles, in addition to 5 mM we have also analyzed AgNPs that were prepared from 2 mM and 10 mM $AgNO_3$ solutions. However, TEM analysis showed no significant change in the shape and the size of the AgNPs. The kinetics of AgNPs production showed that more than 90% of the particles were formed within 18 h of reaction, and were saturated at 24 h (Figure 1c). This observation suggested that the reduction

  

of silver ions and subsequent induction and formation of AgNPs was exponential. Furthermore, a detailed analysis of the silver resistance and AgNP production was carried out in *Morganella* sp. When grown in different concentrations of $AgNO_3$, the bacterium was able to grow in up to 0.5 mM $AgNO_3$. However, the growth rate decreased significantly at 0.6 mM, and no growth was observed above 0.6 mM. Whereas, in a nanoparticle production experiment, the bacterium was allowed to grow up to the late exponential phase, and a different concentration of $AgNO_3$ was then added. The detectable nanoparticles were only found when a minimum of 1 mM $AgNO_3$ was added (Figure 1B). As a consequence, similar experiments have been performed in the closely related bacterium *E. coli* to understand the role of silver resistance in AgNPs production from *Morganella* sp. No extracellular production of AgNPs was detected upon addition of aqueous $AgNO_3$ solution at various growth phases of silver-sensitive *E. coli*, even without any dilution of culture supernatant (Figure 1A, curve f). Comparative studies with our laboratory strains of *Klebsiella* and *Serratia* did not yield any detectable AgNPs (data not shown). This proves the unique ability of *Morganella* sp. to produce extracellular AgNPs. The above-mentioned observations also ruled out the possibility of post-growth respiratory chain reduction of silver ions by silver-resistant *Morganella* sp.[29] Moreover, when the bacterium was challenged with gold ions, which are known to be reduced more easily than silver ions, no gold nanoparticles were detected (data not shown); this indeed proves the specificity of the bacterium to silver ions. It thus seems that the biochemical character that could relate the extracellular AgNPs synthesis in *Morganella* sp. but not in closely related silver-sensitive *E. coli* was its resistance to silver ions. The genes that are believed to be part of the silver-resistant machinery in *Morganella* sp. were highly similar to reported genes. The multiple sequence alignment data of the *silE* homologue from *Morganella* sp. with reported *silE,* which encodes a periplasmic silver-binding protein, revealed that they were 99% similar in nucleotide and amino acid composition; this suggests a similar role in *Morganella* sp. Similarly, *silP* and *silS* homologues were also found to be highly similar to reported *sil* genes at the nucleotide and amino acid sequence level; this also suggests a similar role for these gene homologues in *Morganella* sp. In the case of *Pseudomonas stutzeri*, $Ag^+$ ions were first internalized, and nanoparticles then accumulated inside the cell.[4] Whereas, in case of *Morganella* sp. the AgNP formation occurred extracellularly. Moreover, the experiment with only culture supernatant has also shown the formation of AgNPs. Hence, with the current experimental evidence, we speculate that an extracellular microenvironment has been created by *Morganella* sp. wherein several silver-specific proteins, possibly from the silver-resistance machinery, are secreted outside the cell during the growth. These proteins might specifically interact and allow the reduction of $Ag^+$ ions, which subsequently leads to formation and stabilization of AgNPs (Figure 6). The presence of amide II and amide I bonds on the surface of AgNPs in the FTIR spectroscopic analysis validates the involvement of such silver-specific proteins in the overall synthesis process (Figure 3B). Elucidation of the silver-resistance mechanism was highly recommended in AgNP synthesis from silver-resistant microorganisms,[4,19] however, no information is available to date. At this point, it seems difficult to assign a specific role for *sil* gene homologues in AgNPs synthesis. We strongly believe, however, that understanding the silver-resistance mechanism at the phenotypic and genotypic levels is a significant step towards understanding the overall biochemical mechanism of AgNPs synthesis in *Morganella* sp.

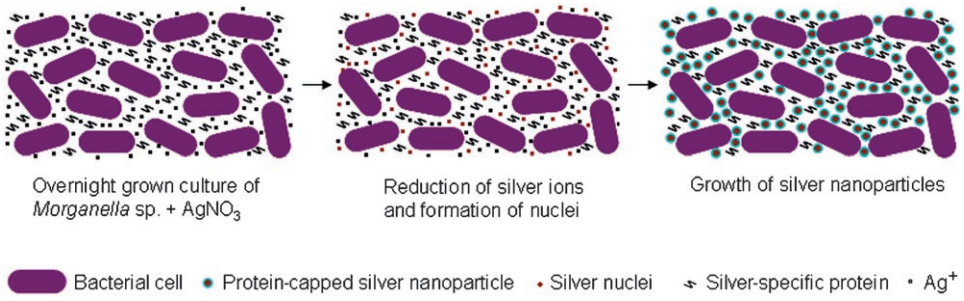


**Figure 6.** Schematic representation of silver nanoparticle formation in an extracellular microenvironment that was created by *Morganella* sp.

## Conclusions

In this study, extracellular synthesis of AgNPs has been shown from silver-resistant *Morganella* sp. TEM and HRTEM analysis showed that most of the particles were spherical in shape, and have characteristic d spacing for the [200] plane. XRD and XPS analysis showed that nanoparticles were crystalline and metallic in nature, respectively. Analysis of biomolecules that are associated with the surface of nanoparticles by FTIR spectroscopy revealed the presence of protein. The extracellular bacterial synthesis of AgNPs has many advantages and might be an excellent means of developing an ecologically friendly protocol. This would be possible only when we understand the biochemical and molecular mechanism of extracellular synthesis of AgNPs in a clear way. The common biochemical character in all previous reports where microorganisms have been utilized for this purpose was silver resistance. Thus as a step towards understanding the mechanism of synthesis of AgNPs, silver resistance has been studied in *Morganella* sp. at the phenotypic and genotypic level. The bacterium was highly resistant to silver cations, and was able to grow in the presence of $AgNO_3$ concentrations higher than 0.5 mM. Three major gene homologues of silver-resistance machinery have been identified from *Morganella* sp. The gene homologue of *silE* was found to be highly similar to reported *silE*, which encodes a silver-binding

  

protein. *silP* and *silS* gene homologues also showed a high percentage similarity with reported silver-resistance genes. It indicated that silver-resistance machinery might have similar role to play in *Morganella* sp. and could be associated with AgNP synthesis. Moreover, the absence of such activity in closely related bacterial genera reinforced the uniqueness and specificity of this bacterium to silver cations. The current investigation is focused on understanding the detailed biochemical and molecular mechanism by studying each gene of the silver-resistance machinery and its product, and to show its probable functional role in silver resistance and extracellular AgNP production in *Morganella* sp.

## Experimental Section

**Extracellular synthesis of AgNPs from *Morganella* sp.**: The bacteria were grown in a 500-mL Erlenmeyer flask that contained LB broth (100 mL) without added NaCl at 37 °C in a shaker incubator set at 200 rpm for 15 h. An aqueous solution of $AgNO_3$ was added at a final concentration of 5 mM to this culture suspension. The flask was then incubated in the dark at 37 °C in shaker incubator at 200 rpm, and the reaction was carried out for 20 h. The extracellular synthesis of AgNPs was monitored by visual inspection of the culture flask for a change in color of culture medium from clear light-yellow to brown, and by measurement of the UV/Vis spectra. All samples for UV/Vis spectra measurement and TEM analysis were prepared by centrifuging an aliquot of culture supernatant (1.5 mL) at 3000 rpm (Eppendorf centrifuge 5810R with a Fixed-angle rotor FA-45-30-11) for 6 min at 25 °C. The supernatant thus obtained was a clear brown homogenous suspension of AgNPs. All samples were diluted 10 times in all experiments in which UV/Vis spectra were measured. From a grown bacterial culture (100 mL), cells were harvested by centrifugation at 3800 rpm (Eppendorf centrifuge 5810R with a Swing-bucket rotor A-4-81) for 20 min at 25 °C. $AgNO_3$ was added to the resultant supernatant to a final concentration of 5 mM, and the solution was incubated under similar conditions in a culture suspension flask. The bacterium was routinely maintained on LB agar slant at 37 °C and preserved in glycerol stock solutions at −80 °C.

**Characterization of AgNPs**: UV/Vis spectroscopy measurements of culture supernatants were carried out on a JASCO dual-beam spectrophotometer (model V–570) that was operated at a resolution of 2 nm. TEM measurements were performed on a JEOL model 1200EX instrument that was operated at an accelerating voltage of 120 kV. TEM measurements of the images in Supporting Information were performed by using FEI-Technai-F30, 300 KV, HRTEM instrument. TEM images were taken by drop coating AgNPs on a carbon-coated copper grid. The HRTEM measurements of purified AgNPs were also carried out on the same carbon-coated copper grids on a JEOL-JEM-2010 UHR instrument that was operated at a lattice–image resolution of 0.14 nm. The XRD pattern measurements of drop-coated films of the AgNPs on glass substrate were carried out on a Philips PW 1830 instrument that was operated at a voltage of 40 kV and a current of 30 mA with Cu $K_\alpha$ radiation. The culture supernatant that was obtained 20 h after the addition of aqueous $AgNO_3$ solution was centrifuged at 3000 rpm for 5 min prior to harvesting bacterial cells; the supernatant was filtered through a 0.22-μm filter, and centrifuged again at 10000 rpm for 15 min to precipitate AgNPs (Eppendorf centrifuge 5810R with a fixed-angle rotor F45-30-11). The resultant pellet was washed vigorously twice with deionized, distilled water for FTIR spectroscopic analysis. A FTIR spectrum of the purified AgNPs was recorded on a Perkin–Elmer Spectrum One instrument at a resolution of 2 $cm^{-1}$. Samples for FTIR were prepared by crushing purified AgNPs with KBr pellets. XPS measurements of films of AgNPs that were cast on to Cu substrates were carried out on a VG MicroTech ESCA 3000 instrument at a pressure lower than $1\times10^{-9}$ Torr. The general scan and C 1s and Ag 3d core level spectra were recorded with unmonochromatized $Mg_{K\alpha}$ radiation (photon energy = 1253.6 eV) at a pass energy of 50 eV and electron take-off angle (angle between electron emission direction and surface plane) of 60°. The overall resolution was ~1 eV for the XPS measurements. The core level spectra were background corrected by using the Shirley algorithm, and the chemically distinct species were resolved by using a non-linear least-squares fitting procedure.[30] The core level binding energies (BE) were aligned with the adventitious carbon binding energy of 285.0 eV. The EDAX measurements were performed on a JEOL JSM-6360A analytical scanning electron microscope. The sample was prepared on a cleaned silicon wafer by drop-coating purified AgNPs.

**Plasmid constructs and polymerase chain reaction**: Chromosomal DNA was isolated by a standard phenol/chloroform extraction method.[31] The 1.5-kb sequence of 16S rRNA gene was amplified by PCR by using universal eubacteria-specific primers and sequenced as described earlier.[32] Nearly 0.43 kb of the *silE* homologue, 2.6 kb of the *silP* homologue, and 1.5 kb of the *silS* homologue were amplified by using BD Advantage 2 PCR Enzyme System (BD Biosciences-Clontech, Mountainview, CA, USA) that consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing and extension at 68 °C for 3 min and final extension at 68 °C for 10 min. The PCR products that were obtained were subsequently cloned into pGEM-T vector (Promega, Madison, WI, USA) by following manufacturers instruction, and transformed in *E. coli* DH5α.[31] Plasmid DNA preparation from all the positive clones that carried the gene of interest was carried out by a miniprep kit (Applied Biosystems) by following the manufacturers instructions. The sequencing of all *sil* homologue constructs of the pGEM-T vector was carried out by using the vector-specific M13F (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) and M13R (5′-TCA CAC AGG AAA CAG CTA TGA C-3′) primers by a DNA Analyzer 3730 by using Big Dye terminator kit (Applied Biosynstems).

**Bioinformatics analysis of nucleotide sequence of 16S rRNA gene and *sil* homologues**: The analysis of all the sequences was performed using blastn, blastx and tblastx programs of NCBI. 16S rRNA gene sequence was analyzed by using the blastn program on the NCBI server; it searches the nucleotide sequence query against the nucleotide database. The *sil* gene homologues were also analyzed by using the blastn program at the NCBI server, but being a protein-coding nucleotide sequence, it was also analyzed by the blastx program, which searches translated nucleotide queries against the protein database and tblastx programs, which searches translated queries against the translated database at the NCBI server. The multiple nucleotide and amino acid sequence alignments were performed by using CLUSTAL W program at the European BioInformatics server (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom) (http//:www.ebi.ac.uk/clustalw) to determine the similarity value.

  

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